REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow. This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

I. Introduction

Applicants appreciate the courtesy extended by the Examiner in conducting a telephone interview with Stephen Maebius, Leon Radomsky and Christine Flynn on January 25, 2006. The following issues were discussed.

Claims 1-143 are requested to be cancelled. Claims 144-157 are being added. Support for claims 144-153 may be found on pages 21-27 and 39-56 of the specification and in the originally filed claims. No new matter was added. After amending the claims as set forth above, claims 144-157 are now pending in this application.

The term "protein" of claim 144 finds support on page 4, line 2, page 11, line 9, page 21, line 16, page 23, lines 14-16, page 47, line 29, the section starting on page 48, line 22 to page 49, line 16, page 53, lines 13 and 29, and page 54, line 7 of the specification. Additional mention of proteins can be found on pages 14-16 of the specification.

The term "synthetic" in the claims means "non-naturally occurring" or "engineered" as would be understood by one of ordinary skill in the art from the use of this term in present specification and from a dictionary definition of this term. This term finds support throughout the specification, such as on page 9, line 24, and page 20, line 24.

During the interview, the examiner indicated that claims 147-151 and 154-157 would be withdrawn from consideration. Applicants request that under the rejoinder procedure of MPEP § 821.04, these claims be rejoined with claim 144 upon allowance of claim 144.

II. The Objection To Specification Should Be Withdrawn

The specification was objected to as failing to provide a proper antecedent basis for the claimed subject matter. Applicants submit that the cancellation of claims 133-143 moots the objection to the specification. Applicants respectfully request that the objection be withdrawn.

III. Section 112, Paragraph 2 Rejections Should Be Withdrawn

Claims 133-141 were rejected under § 112, ¶ 2 as being indefinite. Applicants submit that the cancellation of claims 133-141 moots this rejection. Applicants respectfully request that the rejection be withdrawn.

IV. Double Patenting Rejections Should Be Held in Abeyance

Claim 133 has been rejected for double patenting. Claim 133 has been cancelled. This is believed to moot the double patenting rejections. To the extent the double patenting rejections still apply to claims 144-157, Applicants respectfully request that the rejection be held in abeyance for the following reason.

MPEP 804(B) states:

> The "provisional" double patenting rejection should continue to be made by the examiner in each application as long as there are conflicting claims in more than one application unless that "provisional" double patenting rejection is the only rejection remaining in one of the applications. If the "provisional" double patenting rejection in one application is the only rejection remaining in that application, the examiner should then withdraw that rejection and permit the application to issue as a patent, thereby converting the "provisional" double patenting rejection in the other application(s) into a double patenting rejection at the time the one application issues as a patent.

The other applications used in the double patenting rejections have not issued as patents yet. Therefore, the examiner is requested to maintain the provisional double patenting rejection in abeyance.

If the other applications used in the double patenting rejections have not yet issued as patents when the other rejections are withdrawn in the present application and the double patenting rejection is the only rejection remaining in the present application, then the examiner is requested to withdraw the double patenting rejection and permit the present application to issue as a patent, as required by MPEP 804(B).

If one of the other applications used in the double patenting rejections issues as a patent before the present application, then applicants will consider submitting a terminal disclaimer if such a disclaimer is warranted at the time.

V. Prior Art Rejections Should Be Withdrawn

Claims 133-141 claims have been rejected under § 102 as being anticipated by Naik, Puentes, Sakaguchi, Whaley or Warne. Claims 133-141 have been cancelled rendering the rejection moot.

Applicants submit that claims 144 to 157 are not anticipated by any one of the five references used to reject the claims in the Office Action.

A. Naik is not prior art

The Naik reference is not prior art because it was published after the filing date of the present priority provisional application 60/411,804 (the "'804 application").

The present application claims priority to the '804 application. The '804 application was filed on September 18, 2002. The '804 application supports presently pending claims 144-153 under 35 U.S.C. 112, first paragraph (see paragraphs [0041] – [0050] and [0085] – [0120] of the '804 application). Thus, claims 144-153 of the present application should be afforded the September 18, 2002 effective filing date.

The Naik article was published on-line on October 27, 2002, as noted on the first page of the article under the title. Thus, the Naik article is not prior art with respect to the present application because it was published after the September 18, 2002 effective filing date of claims 144-153 of the present application.

During the interview, the examiner agreed that Naik is not prior art and indicated that this rejection would be withdrawn.

B. Puentes

Claim 144 recites a metal nanoparticle which is bound to a peptide or protein which selectively binds to the metal nanoparticle. In contrast, Puentes does not teach a peptide or protein. Puentes teaches to use colloidal chemistry to form nanocrystals. During the interview, the examiner indicated that this rejection would be withdrawn.

C. Sakaguchi

Claim 144 recites a metal nanoparticle bound to a synthetic peptide or a synthetic protein which selectively binds to the metal nanoparticle. In contrast, Sakaguchi teaches using RS-1 bacteria to form metal oxide particles. Sakaguchi does not teach or suggest that the bacteria contains the claimed synthetic peptide or protein which selectively binds to the metal nanoparticle. Thus, Sakaguchi does not teach the claimed synthetic peptide or protein which selectively binds to the metal nanoparticle.

D. Whaley

Claim 144 recites a metal nanoparticle bound to a peptide or protein which selectively binds to the metal nanoparticle. In contrast, page 665 of Whaley discloses peptides which selectively bind to semiconductor materials. Specifically, GaAs, InP and Si mentioned on page 665 of Whaley are all semiconductor materials, not metal materials. Even the title of the Whaley article states that the peptides have a semiconductor binding selectivity. Whaley does not disclose peptides with a metal binding selectively.

During the interview, the examiner indicated that column 1 on page 665 of Whaley contains a sentence which states that "Peptides with limited selectivity for binding to metal surfaces and metal oxide surfaces have been successfully selected." This sentence refers to the two articles by Brown, which are listed as references 10 and 11 in the Whaley article.

The Brown articles are enclosed with this response. The Brown articles do not disclose peptides which are bound to metal nanoparticles, as recited in claim 144. The Brown article from Nature Biotechnology journal describes the use of a conventional metal powder having a particle size of 1.5 microns (see first paragraph in column 2 on page 272 of the Brown article). This metal powder is clearly not a nanoparticle powder.

The Brown article from the Proc. Natl. Acad. Sci. journal describes the use of a conventional metal oxide powder obtained from a commercial vendor (see first paragraph in column 1 on page 8652 of this Brown article). This metal oxide powder is clearly not a metal nanoparticle powder.

Likewise, the first paragraph of page 665 of the Whaley articles does not disclose peptides which are bound to metal nanoparticles. Therefore, neither the Whaley article nor the Brown articles teach or suggest a peptide or protein bound to metal nanoparticles, as recited in claim 144.

Next to the last paragraph of Page 667 of Whaley discloses tagging G12-3 clones with gold nanoparticles. However, as explained on page 667 of Whaley, the G12-3 clones have selective binding for the GaAs semiconductor material, but not for the gold nanoparticles. The gold nanoparticles are merely used as a tag or label to enable the detection of the G12-3 clones which are bound to the GaAs substrate. Specifically, the section called "Antibody and gold labeling" on page 668 of Whaley states that the gold nanoparticles were coated with streptavidin to form a label. The streptavidin was bound to the biotin conjugated phage through a biotin-streptavidin interaction to form peptide-peptide binding rather than a selective peptide-metal nanoparticle binding of claim 144. Furthermore, as is well known in the art, streptavidin non-selectively binds to most materials. Thus, the streptavidin was not selectively bound to the gold nanoparticles. In contrast, claim 144 requires selective binding between the nanoparticle and the peptide or protein.

Thus, the peptides of Whaley do not have selective binding affinity for metal nanoparticles, as recited in claim 144.

Furthermore, with respect to claim 147, Whaley does not teach that the peptide selectively nucleates metal nanoparticles from a solution, as recited in claim 147. The last paragraph of page 667 of Whaley appears to discuss peptides with a binding affinity for pre-manufactured semiconductor nanoparticles, rather than peptides which can nucleate metal nanoparticles, as recited in claim 147.

E. Warne

Warne teaches using protein shells prepared from native ferritin to form metal grains inside the shells. However, as noted in the last paragraph in the first column of page 3009 of Warne, the empty protein is merely used as a reaction vessel. Thus, the protein of Warne is not a synthetic protein which selectively binds to the metal nanoparticle.

Furthermore, the last paragraph in the first column of page 3009 of Warne also states that the empty protein shell prepared from ferritin could be used to synthesize non-metal nanoparticles, such as cadmium sulfide semiconductor nanoparticles. Therefore, this is clear evidence that the protein shell does not selectively bind to metal nanoparticles, because the protein shell can be used to form metal as well as non-metal nanoparticles.

Therefore, Warne does not teach or suggest a metal nanoparticle bound to a synthetic peptide or protein which selectively binds to the metal nanoparticle, as recited in claim 144.

VI. Conclusion

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to

Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 1/30/06

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Metal-recognition by repeating polypeptides

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Attachment of proteins to metal surfaces has the potential to improve our understanding of protein adhesion and has applications in sensor technology. Repeating polypeptides able to bind to metallic gold or chromium were selected from a population of approximately 5 million different polypeptides. Each polypeptide contained several direct repeats of identical peptide units 14 or 28 amino acids long. The metal-recognizing polypeptides were found to retain their binding properties when freed from the protein used to select them. One gold-binding polypeptide's avidity for gold was found to be dependent on the number of repeats and the presence of salt.

Keywords: biosensors, peptide display, LamB, *Escherichia coli*, gold, chromium

Understanding the mechanisms by which proteins recognize surface features is a central goal in biology. Metal surfaces provide a model system to study surface recognition by proteins. One genetic approach used to analyze the recognition properties of proteins is peptide display[1]. In these approaches, large populations of random peptides are displayed on the surface of either beads, bacteriophage, or bacteria in such a way that each particle displays peptides of a single amino acid sequence. The population of particles are exposed to the target surface and those that recognize the surface and adhere are recovered and analyzed. Short peptides, however, can often attain multiple conformations, reducing the concentration of the active form[2]. Polymerizing short peptides could change their properties, resulting in a more rigid structure as when monomers are polymerized to form a plastic or when hexapeptide units are repeated to form silk[3]. Thus, the polypeptides produced would be large enough to encode their own folding information while retaining a low complexity. Low complexity should aid in analyses leading to modeling and eventual manipulation of the structure of the folded polypeptide. Large populations of repeating polypeptides have been constructed and repeating polypeptides that were able to adhere avidly to, and distinguish, metal surfaces were isolated.

Results

Construction of repeating polypeptide populations. The repeating polypeptides were displayed on the surface of the bacterium *Escherichia coli* as part of the maltodextrin porin, LamB. The promoter for *lamB* was replaced with P*tac*[4] and the structural gene modified to accept the synthetic oligonucleotides encoding the repeating polypeptides between codons 155 and 156. The oligonucleotides encoding the repeating polypeptides were synthesized by a rolling-circle method using the template oligonucleotides (Fig.1). Three different libraries were prepared and pooled to generate a population of approximately 5×10^6 clones.

Isolation of metal-binding polypeptides. Bacteria that adhered to gold were selected by serial enrichment. For comparison, a parallel enrichment was conducted for bacteria that adhered to chromium. After each cycle of enrichment, aliquots of the enriched populations were stored. After several cycles of enrichment, plasmid DNA was prepared from the stored aliquots and the size distribution of the population monitored by PCR using primers complementary to vector sequences flanking the insertion site[5]. Since the number of times each random oligonucleotide was repeated varied from clone to clone, the size distribution of the inserted oligonucleotides reflects the complexity of the population. After nine cycles of enrichment with gold, and after six cycles of enrichment with chromium, the size distribution of the enriched population diverged from that of the initial population. The inserts from the enriched populations were recloned into the alkaline phosphatase expression vector, pSB2991. This transferred any potential metal-binding polypeptide from the insoluble LamB protein to a soluble periplasmic protein, facilitating further analysis.

From the gold-binding enrichments, 14 chimeric alkaline phosphatase proteins were examined by pulse-labeling with ^{35}S-methionine. Of the 14, seven bound to gold. Six of the gold-binding proteins were metabolically stable enough to examine further and one, encoded by pSB3008, was produced in high yield. From the chromium-binding enrichments, six chimeric alkaline phosphatase proteins were examined by pulse-labeling with ^{35}S-methionine. Of these six, four bound to chromium and all were metabolically stable enough to examine further. Of the four chromium-binding proteins, one was encoded by two separate circular templates and was not examined further. Steady-state labeling with ^{35}S-sulfate was used to further characterize the binding behavior since it allows determination of the molar concentrations of proteins.

The selected repeating polypeptides bound avidly to the target metals (Table 1). The chimeric alkaline phosphatases labeled with ^{35}S-sulfate were added to the binding assays at a concentration of only 0.1 nM. Even at this low concentration, a substantial fraction of the added protein adhered to the metal powder. Pulse-labeling with ^{35}S-methionine does not allow a direct determination of protein concentration.

template oligonucleotide 1, library A:

5'	GCT	CTG	NNK	NNK	NNS	GYT	NNK	NNS	CTG	NNK	NNK	NNS	ATG	CAT 3'
	A	L	X	X	X	V/A	X	X	L	X	X	X	M	H

template oligonucleotide 2, libraries B and C:

5'	CC	GGG	ATG	NNS	NNS	NNS	NNS	NNS	NNS	NNS	NNS	NNS	SAG	GCG	ACT	C 3'
	P	G	M	X	X	X	X	X	X	X	X	Q/E	A	T		

Figure 1. The nucleotide and inferred amino acid sequences of the templates used.

Table 1. Deduced amino acid sequences and binding behavior of selected clones

	Library/ repeats	Fraction bound			
		Chromium		Gold	
		EDTA'	total	untreated	HF-treated
Gold-binding:					
pSB3004: alVPTaHRIDGNmh	A/6			.16*	.20*
pSB3071: alPRGvYKIDSNmh	A/9			.52*	.36*
pSB3006: pgmKASKSMRNqatpgmPSSLDLTWqat	C/3.5			.25*	.75*
pSB3081: pgmKMRLSGAKeatpgmSTTVAGLLqat	C/3.5			.14*	.66*
pSB3073: pgmIHVQKTAVqatpgmVNLTSPVKqat	C/6.5			.25*	.58*
pSB3008: first 2: alDSPaGCISFSmh + last 7: MHGKTQATSGTIQS	A/2 A-inv/7	.17	.72	.25	.70
pSB3008 derivatives:					
pSB3127: MHGKTQATSGTIQS	A-inv/9				.89
pSB3053: MHGKTQATSGTIQS	A-inv/7	.13	.66	.24	.91
pSB3055: MHGKTQATSGTIQS	A-inv/6			.08	.79
pSB3057: MHGKTQATSGTIQS	A-inv/5			.01	.52
Chromium-binding:					
QHQK					
pSB3084: pgmDRQQHQSKqat	B/5		.57*		
pSB3088: pgmYNQHQKTKeat	B/5	.58	.92	.03	
pSB3089: pgmDHQKPLGKqat	B/6	.39	.70	.01	.12
Others:					
pSB2991 (vector): no insert	0	.07	.07	<.01	<.01
pSB3103 (random): alRRDvNCIGASmh	A/6		.46	.03	

Amino acids presented in lower case were those encoded by the defined sequences. The values indicate the fraction of chimeric alkaline phosphatase added to the binding assay that bound to the indicated metal powder. *Alkaline phosphatase was labeled with ^{35}S-methionine. Absence of an asterisk indicates the alkaline phosphatase was labeled with ^{35}S-sulfate and was added to the binding assay at a concentration of 0.1 nM alkaline phosphatase monomer.

The gold-binding polypeptides could be divided into two classes after measuring binding to gold that had been treated with hydrofluoric acid to remove surface impurities[6]. One class contained those whose binding was increased by treating the gold with hydrofluoric acid, and the other class contained those whose binding was not increased. Within each class, the sequences showed some similarity. The class whose binding was not increased, those encoded by pSB3004 and pSB3071, contained the sequence (R/K)LD-N. No obvious consensus sequence appeared among those whose binding was increased, pSB3006, SB3008, pSB3073, and pSB3081, but they were all rich in the hydroxyamino acids, serine and threonine. It is likely that the hydrofluoric acid only partially cleaned the gold surface since the treatment used here was less rigorous than that described[6]. Thus, the polypeptides encoded by pSB3006, SB3008, pSB3073, and pSB3081 recognized gold itself, and the polypeptides encoded by pSB3004 and pSB3071 recognized a surface contaminant that was partially removed.

The consensus sequence for chromium-binding was clear, QHQK, suggesting they displayed a property distinct from the rest of the population. Since all of the chimeric alkaline phosphatases bound to chromium in the standard binding assay (Table 1), including that encoded by a random clone, pSB3103, how could pSB3084-pSB3089 have been selected? The repeating polypeptides were selected as part of the maltodextrin porin, an outer membrane protein. Since the lipopolysaccharide component of the outer membrane is a strong chelator[7], the selective environment could have mimicked the presence of EDTA. The repeating polypeptides selected as chromium-binders were more resistant to EDTA than those selected as gold-binders (Table 1). The surface features of the chromium powder recognized by the repeating polypeptides was not investigated.

Characterization of the gold-binding polypeptide encoded by pSB3008. An expected amino acid motif among the gold-binding polypeptides was pairs of cysteines forming disulfide bridges[8]. Two cysteines were encoded by pSB3008, one in each of the first two repeat units. pSB3008, however, contained a large inversion. The first two repeats, those encoding the cysteines, were present in the intended orientation, followed by seven repeats in the inverted orientation. Removal of the first two repeats generated pSB3053 whose product bound to gold with the same avidity as that of pSB3008 (Table 1). This indicated the binding portion encoded by pSB3008 resided in the last seven repeats and thus the cysteines were not contributing to the gold-binding site. Furthermore, the presence of cysteines did not result in binding by the product of pSB3103, a clone in *phoA* derived from a random member of the repeat library.

Binding to the gold powder used here, with no further treatment, saturated with only 36 fmols of pSB3053 encoded alkaline phosphatase per mg gold. Based on the dimensions of alkaline phosphatase[9], 1 mg of gold powder has sufficient surface area to be coated by a maximum of 10 pmols of alkaline phosphatase. Treating the gold with hydrofluoric acid increased the binding capacity of the gold to approximately 1 pmol of pSB3053 encoded alkaline phosphatase per mg of HF-treated gold (740 fmols to 0.9 mg, and 390 fmols to 0.2 mg), or about 10% of the maximum that could be accommodated. One of the control mutants, the chromium-binding alkaline phosphatase encoded by pSB3089, saturated at approximately 12 fmols/mg of HF-treated gold, or about 1% as much as the alkaline phosphatase encoded by pSB3053.

The binding of pSB3053 encoded alkaline phosphatase to HF-treated gold was analyzed by a modified Scatchard method[10,11] to address the condition where a substantial fraction of the substrate is bound. This analysis yielded a K_d of 150 pM which is consistent with the binding data (Table 1).

Removal of additional repeats from pSB3008, beyond the first two, reduced the avidity of the resulting alkaline phosphatases for gold (Fig. 2, Table 1), demonstrating that the binding avidity was dependent on the number of repeats. Increasing the number of repeats to nine did not result in a detectable increase in binding but the assay used was probably close to saturation with the product of seven repeats.

PCR analysis indicated that the inversion in pSB3008 arose during the selection procedure. PCR analysis of the enriched population using an oligonucleotide complementary to the repeat unit of pSB3008 found the inserted oligonucleotides were present in both orientations, but PCR analysis of the initial population found the inserted oligonucleotides present only in the intended orientation.

An 8-bp sequence, CATGCATG, could have permitted the inversion to occur and the sequence predicted to be flanking the 3′ end of the insert prior to inversion shows good homology with DNA gyrase cut sites[12]. The occurrence and selection of a rearrangement such as that in pSB3008 indicates the complexity of populations such as these may differ from that predicted by a simple arithmetic analysis.

Two properties distinguished the binding of pSB3053 encoded alkaline phosphatase to gold from the reported binding of other proteins to gold. In the case of colloidal gold, the binding of proteins requires low salt and is inhibited by salt[13]. The binding of pSB3053 encoded alkaline phosphatase, however, was stimulated by salt. The maximum binding to gold required approximately 0.1 M KCl, at which concentration more than 10 times as much pSB3053 encoded alkaline phosphatase bound as in the absence of added salt. The second property which differed from that of other proteins was the avidity for gold. pSB3053 encoded alkaline phosphatase, unlike most *E. coli* proteins, bound efficiently to gold (Fig. 2). This efficient binding occurred even at low concentrations of the pSB3053 encoded alkaline phosphatase. This high efficiency of binding was observed as the concentration of pSB3053 encoded alkaline phosphatase approached that necessary to saturate the binding experiments. In contrast, the binding of IgG to gold requires a greater than 500-fold molar excess to saturate[14].

If the binding behavior of the repeating polypeptides was independent of the protein used to display them, then the repeating polypeptides themselves should bind to gold. To test this, the gene for alkaline phosphatase was removed from the repeating oligonucleotides. The peptides encoded by pSB3143 that bound to gold were only synthesized in the presence of IPTG. The shorter peptides seen below the full-length repeating polypeptide are probably the products of partial proteolysis. A different, faster growing strain was used in this experiment because *phoA*-deleted plasmids were not recovered with S2157. The larger peptide probably retained its signal sequence. The chromium-binding peptide encoded by pSB3142 bound very weakly to gold (Fig. 3).

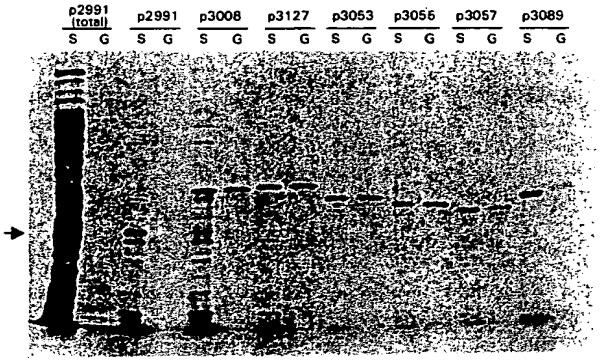

Figure 2. Gold-binding behavior of chimeric alkalines phosphatases labeled with ^{35}S-sulfate. The samples were either transferred directly to SDS sample buffer (S), or first bound to HF-treated gold (G). The extracts were released by osmotic shock except for the lanes marked total, which were whole-cell lysates released by sonication and clarified. p2991 refers to extracts from S2157pSB2991, p3008 refers to extracts from S2157pSB3008, etc. The arrow indicates the position of alkaline phosphatase encoded by pSB2991.

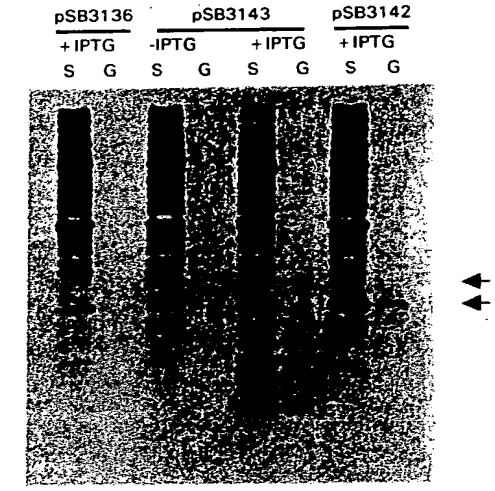

Figure 3. Gold-binding behavior of the isolated repeating polypeptides labeled with ^{35}S-L-methionine in the presence or absence of IPTG. The samples were either transferred directly to SDS sample buffer (S), or first bound to HF-treated gold (G). pSB3136 is derived from the vector, pSB2991. pSB3143 is derived from pSB3127, and pSB3142 is derived from pSB3089. The positions of the mature, full-length repeating polypeptides encoded by pSB3143 and pSB3142 are indicated by the upper and lower arrows, respectively.

Discussion

The use of repeating polypeptides provides several advantages in the analysis of protein interactions. The low complexity of repeating polypeptides renders them amenable to structural analysis in spite of their moderate size. This has been reported for a designed repeating octapeptide[15]. The increased concentration of binding sites present in the repeating polypeptide perhaps allowed the isolation of binding proteins that may not have been found otherwise. For example, few native proteins were able to adhere to gold (Fig. 2 and 3), and the binding activity was dependent on the number of repeat units (Table 1). Although the gold-binding polypeptides were isolated from both libraries A and C, the chromium-binding polypeptides were isolated only from library B. This bias due to the requirement for a defined sequence in the template oligonucleotide may be capitalized upon if the defined sequence encodes a consensus binding sequence identified by other means. Also, repeating polypeptides similar to those described here could possibly be used in the preparation of biosensors. Hybrid proteins containing metal-adhering peptides could be easily immobilized on the metallic sensor surface and permit control of the orientation of proteins relative to the sensor surface by less elaborate procedures than are currently available[16].

Experimental protocol

Strains. S2188, F′*lacI*^{q} Δ*lamB endA hsdR17 supE44 thi1 relA1 gyrA96* Δ*fimB-H::kan*. S2157, as S2188 but Δ*malB101* and Δ(*proC-phoA*). S971, HfrH *lacI*^{q17}.

Plasmids. The LamB expression vector, pSB2267, contains EcoRI and EcoRI-StuI fragments of pAC1[18] replacing the ClaI-BclI fragment of pACYC184[19], and codons 155 and 156 of mature LamB mutated to introduce PstI and XhoI sites. The alkaline phosphatase expression vector, pSB2991, contains the *Ptac* and *phoA* region of pDHB5059 (a gift from Dana Boyd) replacing the HindIII-XbaI fragment of pET19b (Novagen, Madison WI) bearing the *bla* gene of pUC8[20]. PstI and XhoI sites were inserted at a permissive site[21] between codons 6 and 7 of mature alkaline phosphatase. Transformants of pSB2991 derivatives were selected in S2157. pSB3103 is a random clone in pSB2991.

Construction of Libraries. Template oligonucleotides containing 25 random nucleotides and 17 nucleotides of defined sequence (Fig. 1) were circularized by annealing with oligonucleotides complementary to the defined nucleotides and treatment with T4 DNA ligase. The products of this reaction were electrophoresed on polyacrylamide gels, the circular form identified by its resistance to T4 DNA polymerase and purified from polyacrylamide gels. Monomer circles purified from template 1 were used to generate library A. Monomer and dimer circles purified from template 2 were used separately to generate libraries B and C respectively. The purified circular templates were annealed with oligonucleotides whose 3′ portions were complementary to

the defined nucleotides of the circular template and whose 5′ portions included an XhoI recognition site within a primer site for later PCR amplification (rolling circle primer of library A; 5′ CAGCCAGTTGCTCTCGAGGGACAGAGCATGCAT 3′, rolling circle primer of libraries B and C; 5′CAGCCAGTTGCTCTCGAGGGACATCCCGGGAGTCGCCT 3′). The rolling circle primers were extended around the circular template by the Sequenase (US Biochemical, Cleveland, OH) form of T7 DNA polymerase. DNA polymerization was then continued around the circular template many times[22] using a single-stranded DNA binding protein[23], T4 gene 32 protein (Boehringer Mannheim, Germany), to generate the repeating oligonucleotides. An oligonucleotide containing a PCR primer sequence with a PstI recognition site at its 5′ end was annealed to the defined nucleotides now present in the repeating oligonucleotide and extended with the Klenow fragment of *E. coli* DNA polymerase (PstI primer of library A; 5′ GGTTCACAGGCTTGGTCTGCAGGCTCTG 3′, PstI primer of libraries B and C; 5′ GGTTCACAGGCTTGGTCTGCAGGCGACTCCCGGGATG 3′). The repeating oligonucleotides containing PCR primer sites at both ends were amplified by PCR (PstI PCR primer; 5′ GGTTCACAGGCTTGGTCTGCAG 3′, XhoI PCR primer; 5′ CAGCCAGTTGCTCTCGAGGGA 3′). The resulting double-stranded repeating oligonucleotide was digested with PstI and XhoI, cloned into pSB2267 and transformed[24] into S2188.

Varying the number of repeats in pSB3008. The reduction of repeat units from pSB3008 was by partial NsiI digestion and recloning of the NsiI-XhoI fragments into the PstI and XhoI sites of pSB2991. The number of repeats was increased by template jumping[25] but using Vent polymerase (New England Biolabs, Beverly, MA) following partial NsiI digestion. The structural gene for alkaline phosphatase was removed from the repeating units by digestion with XhoI and HindIII, filling in the ends with the Klenow fragment of *E. coli* DNA polymerase, ligating and transforming S971. This added three amino acids after the leucine and glutamate encoded by the XhoI site.

Enrichment procedure. Cultures were established at 30°C in YT broth[26] supplemented with 25 μg/ml chloramphenicol and transcription of the hybrid *lamB* genes induced by 2 mM IPTG. This procedure results in approximately 10,000 copies of the hybrid protein to be displayed on the surface of the bacterium. The induced cultures were diluted into M63 salts[26] containing a final concentration of 75% Percoll (Pharmacia, Uppsala, Sweden). At this concentration, the bacteria were less dense than the solution. Metal was added at a concentration of 0.5–1 mg/ml and the bacteria allowed to adhere at room temperature. Following this incubation, the suspensions were centrifuged, the supernatants with the nonadhering bacteria discarded, and the metal with any adhering bacteria resuspended in YT broth supplemented with 25 μg/ml chloramphenicol. The broth suspensions were incubated overnight, during which the bacteria multiplied under conditions that did not induce expression of the hybrid LamB protein. After each cycle of enrichment, aliquots of the saturated cultures were frozen at -80°C with 15% glycerol and stored for later analysis.

Labeling conditions and preparation of extracts. Labeling with ^{35}S-L-methionine was for 5 min in M63-glucose medium[26] supplemented with 100 μg/ml ampicillin at 34°C following induction with 2 mM IPTG. Labeling with ^{35}S-sulfate was in M63 medium made 100 μM Na_2SO_4 and IPTG was added to 2 mM, 30 min after addition of ^{35}S-sulfate. Chimeric alkaline phosphatases were labeled in S2157 and the isolated repeating polypeptides were labeled in S971. Periplasmic extracts containing the chimeric alkaline phosphatases and the isolated repeating polypeptides were prepared by cold osmotic shock[27].

Binding assays. Binding assays were conducted at room temperature in 1 ml 10mM potassium phosphate, pH 7.0, 0.1M KCl, 1% Triton X-100 (PKT) containing 1 mg metal and 100 fmols alkaline phosphatase monomer. All concentrations reported here were the concentrations of alkaline phosphatase monomers. The suspensions were incubated 10 min with constant mixing. The metal was sedimented by centrifugation for 10 sec in a microfuge. For gold-binding, the supernatant was discarded and the metal resuspended in SDS sample buffer[28]. For chromium-binding, the binding buffer contained 20 μg/ml BSA. After centrifugation of the chromium suspension, 200 μl of the supernatant was aspirated to a fresh tube and 1 ml cold acetone added. The precipitate was recovered by centrifugation, dried, and redissolved with SDS sample buffer. The samples were electrophoresed on 8% polyacrylamide-SDS gels[28] for hybrid alkaline phosphatases, or 15% Tricine-SDS gels[29] for the isolated repeating polypeptides. The fraction of hybrid alkaline phosphatase whose binding was sensitive to EDTA was measured by boiling the chromium pellet with SDS sample buffer made 50 mM in sodium EDTA prior to electrophoresis. Radioactivity was quantified with a phosphor-imager. The molar concentration of ^{35}S-sulfate-labeled proteins was determined by excising bands from gels and scintillation counting.

Source of reagents and HF-treating gold. Gold as a spherical powder having an average particle size of 1.5 μm was obtained from Aldrich (Milwaukee, WI; catalog number 32,658-5). Twenty-milligram aliquots were incubated with 1.5 ml 48% HF overnight[4]. The gold powder was then washed sequentially with H_2O, 0.1M KPO, pH 7.0 and PKT. The gold powder was then resuspended in PKT and used immediately as HF-treated gold. Chromium powder was also obtained from Aldrich (catalog number 26,629-9) and was washed with PKT for use in binding assays, or M63 salts for enrichments.

Acknowledgments

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Genetics

Engineered iron oxide-adhesion mutants of the *Escherichia coli* phage λ receptor

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ABSTRACT ***Escherichia coli*** **able to specifically adhere to iron oxide and not adhere to other metal oxides were constructed by genetic engineering. Concatamers of random oligonucleotides were introduced into a portion of a plasmid-borne *lamB* gene encoding an external domain of the phage λ receptor. Bacteria able to adhere to iron oxide were selected by serial enrichment from the population of plasmid transformants. The concatameric nature of the inserted DNA allows a genetic analysis analogous to exon shuffling. Results of this genetic analysis indicate that in some isolates, part of the binding site is encoded by flanking vector sequences. This strategy may prove generally useful for identifying protein sequences able to recognize specific surfaces.**

A number of experimental approaches have been applied to the production and identification of synthetic RNAs (1, 2), DNAs (3), and proteins (4–9) able to interact specifically with defined substrates. Immunoglobulin cDNA has been used as a means to encode a large array of protein sequences. From these populations catalytic antibodies (4) and antibody fragments able to bind a small molecule (5) have been isolated. Short random peptides of 6 (6, 7) and 15 (8) amino acids have been produced from clones of random oligonucleotides, and random peptides of 5 amino acids have been synthesized chemically on beads (9). The short peptides have allowed the identification of peptides recognized by antibodies and streptavidin.

The isolation of engineered proteins that bind specific substrates and their comparison with natural proteins of similar specificity may facilitate the identification of substrate-binding motifs. In addition, binding sites able to recognize molecules not common in biological systems may be found among synthetic proteins.

Here I report the construction of an *Escherichia coli* library displaying random protein sequences on the outer surface of the bacterium. The strategy used relies on the detailed characterization of the λ receptor (10), especially the results of Hofnung and coworkers (11), who identified a domain of the λ receptor that is external to the bacterium and able to tolerate the insertion of foreign protein, and the observation of Ferenci and Lee (12) that the λ receptor confers on *E. coli* the ability to adhere to starch-Sepharose. From this library I have identified engineered proteins that confer on the bacterium the ability to adhere to iron oxide.*

MATERIALS AND METHODS

Strains and Plasmids. The bacteria used in this study are all derivatives of *E. coli* K-12: S1755, F⁻ *lacI*^{Q} Δ*malB101 endA hsdR17 supE44 thil relA1 gyrA96*; S1918, as S1755 but Δ*fimB–H*::*kan*; S1964, as S1918 but *ara*::Tn*10 mini-tet*; S1995, as S1755 but containing the *malB* allele of SE2016 (13).

The M13 region from pZ150 (14) was introduced into the *lamB* expression vector pAC1 (15) by homologous recombination. Codons representing amino acids 155 and 156 of mature λ receptor were then converted in the *lamB* gene to an *Xho* I restriction site by replacing the *Cla* I–*Ssp* I fragment including this region with synthetic oligonucleotides to generate pSB1649. The control plasmid pSB1819 was recovered from a nearly random library similar to that described below. pSB1819 retains the ability to confer fermentation of maltodextrin on *lamB* strains.

Construction of the Library. Recombinant DNA manipulations and gel electrophoresis were done as described (16) with the modifications described below. The template oligonucleotide is 5′-GGACGCCTCGAG(VNN)$_9$CTCGAGAGCAACAAT-3′. N indicates an equimolar mixture of all four nucleotides, and V indicates an equimolar mixture of A, C, and G. The use of VNN codons prevents the appearance of functional nonsense codons in either orientation in amber-suppressing hosts. The primer oligonucleotide 5′-ATTGTTGCTCTCGAG-3′ was hybridized to the template oligonucleotide, and the primer was extended with Klenow fragment of DNA polymerase. The unincorporated nucleotides were removed by chromatography over Sephadex G25, and the recovered double-stranded oligonucleotide was digested with *Xho* I (New England Biolabs) as per the manufacturer's recommendations at 30°C, releasing an internal 33-base-pair (bp) fragment. The 33-bp fragment was purified by electrophoresis through 16% polyacrylamide gel in 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3 (TBE) buffer at 4°C. A portion of the gel was stained with ethidium bromide, and the band representing the 33-bp internal fragment was excised from the unstained portion. The fragment was eluted from the gel with 10 mM Tris·HCl, pH 8.0/2 mM EDTA/0.15 M NaCl. The eluate was filtered through 0.2-μm nitrocellulose filter, concentrated by ethanol precipitation, and redissolved with 10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.1 M NaCl. The redissolved 33-bp *Xho* I fragment was ligated at various ratios with *Xho* I-digested pSB1649 in 66 mM Tris·HCl, pH 8.0/25 mM NaCl/10 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP/T4 DNA polymerase at 12°C. The ligation products were extracted with phenol, precipitated with ethanol, and desalted over Sepharose 4B before transformation of S1755 by protocol 3 (17).

The oligonucleotide-shuffled libraries were prepared as described above but with the inclusion of the six double-stranded 33-bp oligonucleotides from pSB1972 with each oligonucleotide at a concentration of ≈1.5% of the total oligonucleotides ligated with pSB1649. The oligonucleotides from pSB1972 were prepared by PCR amplification of CsCl-ethidium bromide-purified pSB1972 with upstream and downstream PCR primers and gel purification after *Xho* I digestion.

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Abbreviation: IPTG: isopropyl β-D-thiogalactoside.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M96870–M96877).

Metal Oxides. Iron oxide was purchased as a dark brown aqueous suspension (catalog no. 4200B) from Advanced Magnetics (Cambridge, MA). The following metal oxides were purchased from Aldrich: Fe_2O_3 (catalog no. 20,351-3), Fe_3O_4 (catalog no. 31,006-9), and Cr_2O_3 (catalog no. 20,306-8). Particles of a size suitable for microscopy were prepared by differential sedimentation of metal oxide suspensions in distilled water. All metal oxides were suspended in M63 salts (18) before mixing with bacteria for microscopy.

Enrichment Procedure. Logarithmic-phase cultures of the bacterial population were established at 37°C in yeast extract/tryptone (YT) broth (18) supplemented with ampicillin at 200 μg/ml. Expression of the recombinant λ receptors was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 2 mM and incubating at 37°C for 15 min. The bacteria were recovered by centrifugation and resuspended with buffer A (1 mM D-mannose in M63 salts). The D-mannose was included to reduce nonspecific adhesion by the bacterial fimbrae (19). The bacterial suspension was mixed with iron oxide at 200 μg/ml (Advanced Magnetics) in buffer A, and the mixture was incubated at room temperature for 10 min. The iron oxide particles with adhering bacteria were recovered by placing the culture tube next to a magnet. After the magnetic separation, the liquid and nonadhering bacteria were removed by aspiration, and the culture tube was removed from the vicinity of the magnet. The iron oxide was resuspended with M63 salts and spread on YT-ampicillin agar. After overnight incubation of the agar plates at 37°C, colonies appearing on the surface were pooled, exponentially growing cultures were established, and the induction and enrichment procedures were repeated.

PCR Analysis and DNA Sequencing. Insert regions in plasmid DNA were PCR amplified (20) 15 cycles with Vent polymerase (New England Biolabs) and indicated primers. Primers used were upstream PCR primer 5'-ACATCGATGTTGGCTTCGGT-3', downstream PCR primer 5'-ATCTGCGCTAAACGCACATCG-3' (see Fig. 1), and RRTVK primer 5'-GCGCCGCACTGTTAAGCA-3'. The RRTVK primer is part of the sense-strand sequence of the first oligonucleotide inserted in pSB1972.

The insert region from CsCl-ethidium bromide-purified plasmid DNA was sequenced by the dideoxynucleotide chain-termination sequencing method with upstream and downstream PCR primers and Sequenase (United States Biochemical).

***In Vitro* Adherence to Iron Oxide.** Transformants of S1918 were cultured, and expression of λ receptor was induced as in the enrichment procedure. λ Receptors were prepared by the rapid maltoporin extraction method of Heine *et al.* (21), except sonication rather than incubation with nucleases was used to reduce viscosity, and membrane particles were washed and resuspended with 50 mM Tris·HCl, pH 8/5 mM $MgCl_2$/1% Triton X-100 instead of washing with 20% (vol/vol) ethanol and resuspending in sample buffer. The washed outer-membrane particles were incubated at room temperature for 10 min in 1 ml of M63 salts containing 1% Triton X-100 with or without 100 μg of iron oxide particles (Advanced Magnetics). The iron oxide with any adhering material was recovered by magnetic separation. Any material not associating with the iron oxide was recovered from the supernatant by centrifugation (21). The separated iron oxide and the outer-membrane pellet recovered from the supernatant were both resuspended in gel sample buffer (16), and the entire samples were examined by electrophoresis through 10% polyacrylamide gel in the presence of SDS (16) and then stained with Coomassie brilliant blue.

RESULTS

Construction and Characterization of the Initial Library. To reduce the assumptions regarding both size and amino acid composition of a protein domain able to adhere to iron oxide, various numbers of oligonucleotides 11 codons in length and composed of nearly random sequence were inserted into the *lamB* gene. The insertion site in *lamB*, the gene for the λ receptor, is within a region encoding an external domain found to be particularly plastic in its ability to accept the insertion of foreign protein sequences and allow accurate integration into the outer membrane (11).

The plasmid into which the oligonucleotides were cloned, pSB1649, is a derivative of the *lamB* expression vector pAC1 (15). pSB1649 bears a single *Xho* I site in a portion of *lamB* that encodes the external domain of the λ receptor described above. *lamB* in these vectors is expressed from the *lac* promoter. A transformant population of 2.2×10^6 independent clones was grown and stored under conditions in which *lamB* is repressed. The size distribution of the concatameric insert population was monitored by the PCR amplification (Fig. 1). Lane 2 in Fig. 1*B* shows the products of PCR amplification of the initial population of clones from which the iron oxide-adhering mutant was selected. Each band represents a size class having a particular number of oligonucleotides inserted. The lowest band comigrates with the PCR product of the vector (lane 1) and is the product of the vector religated with no oligonucleotide inserted. The next band is the product of the vector religated with one oligonucleotide inserted, the third band is the product of the vector

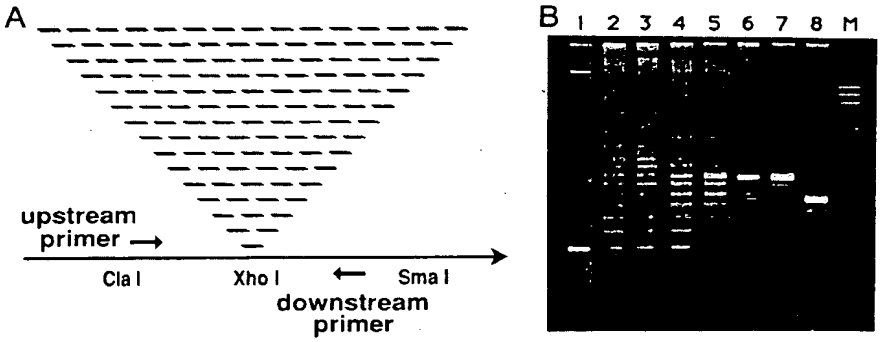

FIG. 1. Structure and PCR analysis of the populations. (*A*) Relative positions of *Cla* I, *Xho* I, and *Sma* I restriction sites and PCR primer sites are indicated in cloning vector pSB1649. The arrow indicates direction of *lamB* transcription. Various numbers of 11-codon oligonucleotides are shown inserted into the *Xho* I site of the vector. (*B*) Products of PCR amplification were electrophoresed in 6% polyacrylamide gel in TBE buffer and stained with ethidium bromide (16). Lanes: 1, vector pSB1649; 2, starting population; 3, four cycles of induction with IPTG transfer to buffer A and return to growth medium; 4, 5, and 6, three, four, and five cycles of enrichment with iron oxide; 7, pSB1972; 8, pSB1971; M. φX174 *Hae* III digest.

religated with two oligonucleotides inserted, etc. The size distribution of the population is very uniform, as evidenced by the relative intensity of the bands, and each size class contains $\approx 10^5$ independent clones. Sensitivity of the heated and cooled PCR amplification products to the single-strand-specific mung bean nuclease indicates the insert population contains a high degree of complexity (data not shown).

Enrichment of Bacteria Able to Adhere to Iron Oxide. Cells able to bind to the surface of iron oxide particles were enriched from cultures of the transformant population. Expression of the engineered λ receptors was induced, and the bacteria were transferred to a suspension of iron oxide particles (see *Materials and Methods*). Adhesion was permitted to occur, and the iron oxide with any adhering bacteria was recovered and transferred to growth medium. The following day, cultures were reestablished, and the induction and enrichment procedure were repeated.

In a control experiment, four cycles of induction, transfer to buffer A in the absence of iron oxide, and outgrowth did not appreciably change the distribution of the insert population, as measured by the PCR assay (Fig. 1). However, clones bearing an insert of 6 oligonucleotides became highly represented after enrichment in the presence of iron oxide (Fig. 1). Ten individual colonies from the fifth cycle of enrichment were purified, and their plasmid DNA was prepared. Restriction analysis of the plasmid DNA indicated that 7 of the 10 DNAs examined contain a 6-oligonucleotide insert—i.e., their *Cla* I–*Sma* I fragment is ≈200 bp larger than that of the vector pSB1649. Of the remaining three plasmids examined, two contain inserts of 3 oligonucleotides and appear identical from restriction patterns with *Bst*X1 and *Age* I (pSB1971 and pSB1977). The final plasmid examined, pSB1978, bears an insertion of 4 oligonucleotides. All seven of the 6-oligonucleotide-bearing plasmids had acquired a single *Bsp*1286 site in the same location, indicating that they are likely to be siblings. One of these plasmids, pSB1972, was sequenced and characterized further.

In a second, independent enrichment from the same starting bacterial population as used in the first experiment, five of six survivors harbored plasmids bearing an insert of 6 oligonucleotides, all five of which contained a single *Bsp*1286 site in the same location as in pSB1972. One member of the second set, pSB1981, was sequenced (see *Materials and Methods*) and found to bear the same sequence as pSB1972. Thus, in two independent enrichments from the same starting population, the same plasmid was recovered.

Adherence to Iron Oxide Is Plasmid Encoded. The strategy used here assumes that the ability to adhere to iron oxide is directed by the plasmid-borne *lamB* gene. This hypothesis was tested by the method of Andrews and Lin (22). In this test, both the vector pSB1649 and the recovered clone pSB1972 are independently transformed into a strain either able to ferment arabinose (S1918) or unable to ferment arabinose (S1964). The transformants are then mixed such that (*i*) one plasmid is in the Ara^+ strain and the other is in the Ara^- strain and (*ii*) the vector-bearing bacteria represent >99.5% of the population. The composition of the mixture was monitored by spreading on the indicator medium, arabinose-MacConkey agar. A single cycle of enrichment with iron oxide increased representation of the strain bearing pSB1972 by 44-fold and was independent of which host bears pSB1972. pSB1971, pSB1977, and pSB1978 were tested only in the Ara^+ strain against the vector in the Ara^- strain. After enrichment, the representation of pSB1971 and pSB1977 transformants increased 34- and 36-fold, respectively. The pSB1978 transformant became enriched only 4-fold and was not examined further.

Transformants harboring pSB1972 were mixed with iron oxide and examined by phase-contrast microscopy. Fig. 2 shows that cells bearing pSB1972 form aggregates in the presence of iron oxide. Neither transformants harboring an unselected control clone pSB1819 or harboring pSB1972 when not induced with IPTG (data not shown) aggregate under these conditions, as determined by phase-contrast microscopy. Transformants harboring the vector pSB1649 behaved similarly to pSB1819 transformants and failed to aggregate with metal oxides (data not shown).

The ability of pSB1972 to confer adherence to specific metal oxides was also examined by phase-contrast microscopy. Fig. 2 shows that pSB1972, but not pSB1819, permits adherence to Fe_2O_3. The weak adherence to Fe_3O_4 by pSB1972 transformants is not significantly different from the background adherence of pSB1819 transformants. The iron oxide used in the enrichments is probably a mixture of Fe_2O_3 and Fe_3O_4. The absence of adherence to Cr_2O_3 indicates the specificity of binding by the pSB1972 *lamB* product to Fe_2O_3.

Oligonucleotide Shuffling. The method used to identify the portion of pSB1972 that encodes the iron oxide-adhering ability is analogous to exon shuffling (23). The individual oligonucleotides composing the insert of pSB1972 were reassorted with a large excess of nearly random oligonucleotides and used to generate two additional libraries (*Materials and Methods*). Shuffled libraries A and B contain 6.1×10^5 and 7.7×10^5 independent clones, respectively. Before enrichment, the shuffled libraries should contain the first oligonucleotide of pSB1972, RRTVKHHVN, randomly located in both orientations among the inserts. PCR analysis of shuffled library A using the RRTVK primer and the upstream and downstream primers (*Materials and Methods*) finds the first oligonucleotide of pSB1972 in the sense orientation equally distributed from 1 to at least 15 oligonucleotides from the 3′ end of the inserts, and in the antisense orientation equally distributed from 1 to at least 14 oligonucleotides from the 5′ end (data not shown).

The shuffled libraries were subjected to four cycles of enrichment with iron oxide, and individual survivors were purified and examined by phase-contrast microscopy for their ability to adhere to iron oxide (Advanced Magnetics). Two of 12 survivors examined from shuffled library A form aggregates with iron oxide: transformants of pSB2065 and pSB2071. Three of 6 survivors examined from shuffled library B form aggregates with iron oxide: transformants of pSB2076, pSB2077, and pSB2078. pSB2077 and pSB2078 are probably siblings because they have the same size insert and acquired *Pml* I and *Sac* I restriction sites.

The sequences encoded by the insert in pSB1972 and the oligonucleotide shuffled decendents are shown in Fig. 3. Of the four different plasmids recovered from the oligonucleotide shuffling experiment, three had acquired the first oligonucleotide from pSB1972. pSB2076 and pSB2077, like pSB1972, contain this oligonucleotide as the first oligonucleotide in the insert. pSB2071 also contains this oligonucleotide but does not contain it as the first in the insert.

Only one of the sequenced plasmids, pSB2077, acquired a second oligonucleotide of pSB1972, so it is difficult to know whether this oligonucleotide contributes to the binding site encoded by pSB2077. None of the oligonucleotides of pSB1972 was recovered in the antisense orientation among the selected clones. pSB2065, which was recovered from shuffled library A, has no oligonucleotide from pSB1972.

The metal oxide-adhesion behavior of pSB2071 and pSB2065 transformants is shown in Fig. 2. pSB2071, like its ancestor pSB1972, allows the transformants to adhere to mixed iron oxide and pure Fe_2O_3 weakly, if at all, to Fe_3O_4, and not to adhere to Cr_2O_3. The unrelated plasmid pSB2065 only confers the ability to adhere to the mixed iron oxide and not to Fe_2O_3, Fe_3O_4, or Cr_2O_3.

In addition to providing the surface receptor for bacteriophage λ, the *lamB* product forms the outer-membrane porin for maltodextrins. When tested on the indicator medium of maltodextrin-MacConkey agar, all of the *lamB* plasmids

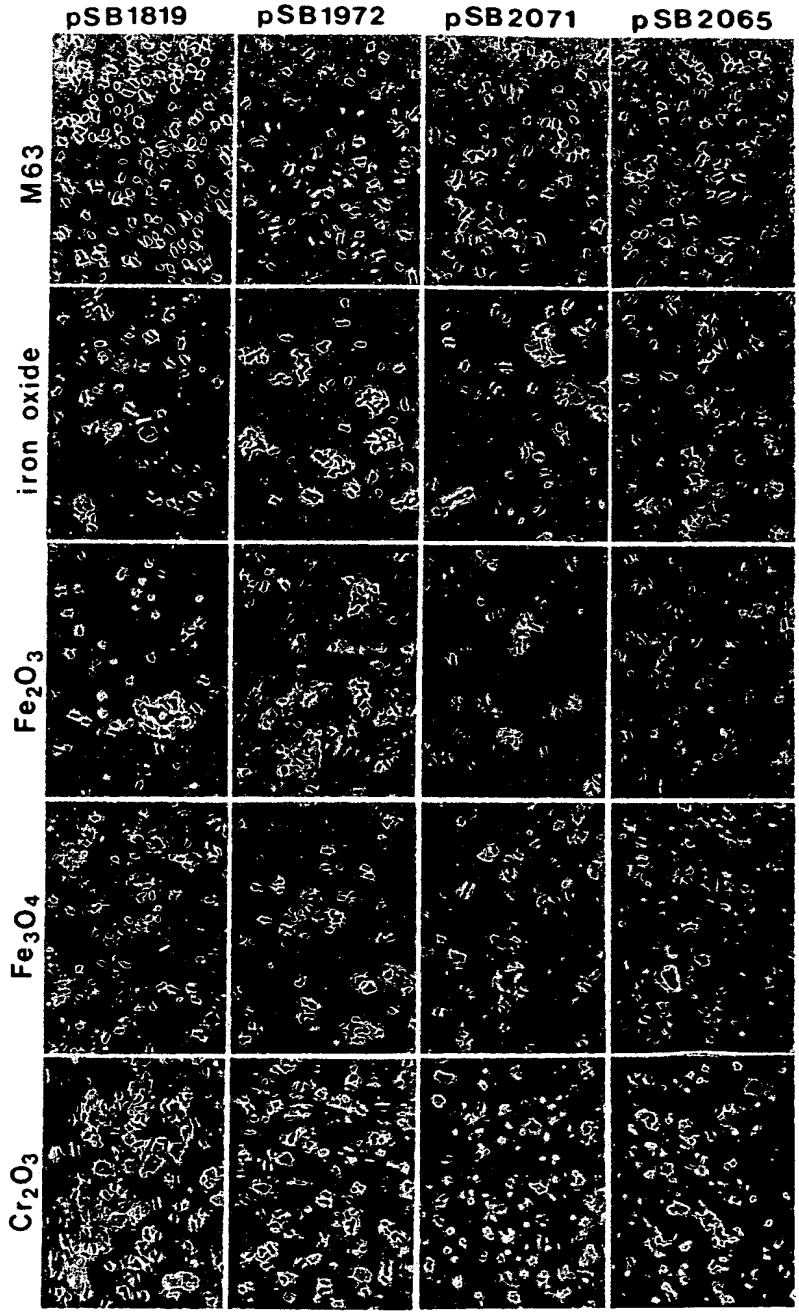

FIG. 2. Adherence to metal oxides. IPTG-induced transformants of S1918 were collected by centrifugation and resuspended with M63 salts. The metal oxides suspended in M63 salts or M63 salts alone were mixed with the resuspended bacteria and examined by phase-contrast microscopy.

described here, but not the control plasmid pBluescript KS(−) (Stratagene), confer the ability to ferment maltodextrins on strain S1995. However, the insert-containing plasmids, unlike vector pSB1649, do not allow bacteriophage λ to infect strain S1995. Thus, the additional protein encoded by the inserts only interferes with adsorption by phage λ and does not interfere with maltodextrin porin function, indicating accurate integration into the outer membrane.

Adhesion of Outer-Membrane Particles to Iron Oxide. The mutant λ receptors confer on whole cells the ability to adhere to iron oxide. To test whether the whole cell is required to observe this adhesion, I prepared outer-membrane particles from cultures induced for expression of the λ receptors (*Materials and Methods*). Fig. 4 shows that the predominant protein in the outer-membrane particles is the λ receptor. After induction with IPTG, very similar amounts of λ receptor accumulate from pSB1649 and pSB2071, and slightly less accumulate from pSB1972 (data not shown). In the absence of IPTG, much less λ receptor accumulates (data not shown). Fig. 4 shows that although the product of the initial isolate pSB1972 causes marginally greater adherence to iron oxide than the product of the vector, the product of pSB2071 causes substantially greater adherence. Fig. 4 also shows that attraction to the magnet requires the presence of iron oxide.

DISCUSSION

This report shows that bacteria able to adhere to a specific material, iron oxide, can be isolated among mutants altered

pSB1972
*TRSSEAGGSSS*LERRTVKHHVNLEDTIAIKEDILEITALARSTLLEERIKPTRLALEITAPKRLPVLESPLELSRQILE
*TRSSEAGGSSS*LERRTVKHHVNLEGGGEMRTGSLEDTAKIHVVKLETRRAQRKRVLE*SNNIYDYTNET* pSB2076
*TRSSEAGGSSS*LERRTVKHHVNLEVKALRPREVLELETEQQTGRLEDTIAIKEDILEASPPPHSVHLE pSB2077
LEQPNSHGSACLERRTVKHHVNLELRMKNPTKNLEGSKMEDNGILEIKEGAVAAKLE*SNNIYDYTNET* pSB2071

pSB1971 LEVAKKPVAMILEHRKRQKTGLLEEKKNTRRVLLE
pSB2065 LEGRIRRSKLRLETHDPSATRGLEKKPPRTAAKLEKKVVRDQASLE

pSB1819 LERIITPREVSLEGHHHTCFAYLECVRAQCTQSLEELHDRMIHALE

FIG. 3. Translated sequences of the inserts described in text. Amino acids of the flanking portions of the λ receptor are italicized. The oligonucleotide recovered most frequently is underlined, as are the glycine and serine preceding that oligonucleotide. The leucines and glutamates encoded by the *Xho* I sites are in boldface type.

in a gene encoding an outer-membrane protein. Furthermore, outer-membrane particles composed predominantly of λ receptor from pSB2071 adhere to iron oxide. The simplest interpretation of the results presented here is that the mutant λ receptors themselves bind to iron oxide. The greater ability of outer-membrane particles containing the λ receptor from pSB2071 to adhere to iron oxide suggests that these particles may have a greater avidity for iron oxide than those containing its "ancestral" λ receptor from pSB1972.

Genetic analysis of the first plasmid characterized, pSB1972, indicates that at least a portion of the RRTVKHHVN peptide contributes to the iron oxide-binding site because the oligonucleotide most frequently recovered in the oligonucleotide-shuffling experiment encodes RRTVKHHVN. The genetic analysis further suggests that part of the binding site is contributed by a portion of the λ receptor amino-proximal to the insert. In pSB2076 and pSB2077, like in their ancestor, pSB1972, the RRTVKHHVN oligonucleotide is the first oligonucleotide in the insert. In pSB2071, however, the RRTVKHHVN oligonucleotide is preceded by another oligonucleotide that encodes a glycine and serine in the same position relative to the RRTVKHHVN oligonucleotide as encoded by *lamB* in the others (Fig. 3).

Transformants of plasmid pSB2071, like transformants of its ancestral plasmid pSB1972, adhere to rust, Fe_2O_3, whether pure or on the surface of magnetite, Fe_3O_4. Transformants of the unrelated isolate, pSB2065, recognize a different material and do not adhere to pure Fe_2O_3 or Fe_3O_4, but only to the mixed iron oxide. This explains the absence of sequence similarity between pSB2065 and the pSB1972 family of inserts.

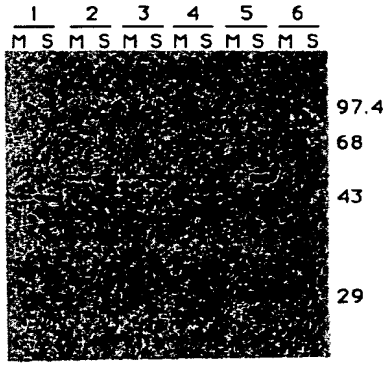

FIG. 4. Association of mutant λ receptors with iron oxide. A polyacrylamide gel testing the association of outer-membrane particles with iron oxide is shown. The material attracted to the magnet is in lanes marked M, and that in the supernatant is in lanes marked S. Separations 1–3 were conducted in the presence of iron oxide, and separations 4–6 were conducted in the absence of iron oxide. Separations 1 and 4 were conducted on a transformant harboring pSB1649, separations 2 and 5 were conducted on a transformant harboring pSB1972, and separations 3 and 6 were conducted on a transformant harboring pSB2071. Numbers at right indicate migration of phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase, having molecular weights of 97,400, 68,000, 43,000, and 29,000, respectively.

Continued application of the strategy used here may identify mutants of the λ receptor able to recognize other surfaces. Mutants described here apparently distinguish the surface of Fe_2O_3 from the surface of mixed Fe_2O_3 and Fe_3O_4. Sets of such mutants may allow the identification of materials by their different adhering properties. Finally, if mutants can be isolated that bind specific soluble molecules, proteins composed of multiple binding sites could, perhaps, function as assemblers of nanomachines (24).

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